UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF TISSUE CULTURE AND HAIRY ROOT PRODUCTION SYSTEMS FOR SOLENOSTEMON SCUTELLARIOIDES (L.) CODD

LE VINH THUC.

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ESTABLISHMENT OF TISSUE CULTURE AND HAIRY ROOT PRODUCTION SYSTEMS FOR *Solenostemon scutellarioides* (L.) Codd

By

LE VINH THUC

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September 2004

Dedicated to my parents

Mr. Lê Văn Khoảnh and Mrs. Trần Thị Kim Đẳng

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

ESTABLISHMENT OF TISSUE CULTURES AND HAIRY ROOT PRODUCTION SYSTEMS FOR *Solenostemon scutellarioides* (L.) Codd

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LE VINH THUC

September 2004

Chairman: **Associate Professor Norihan Mohd. Saleh, Ph.D.**

Faculty: **Biotechnology and Biomolecular Sciences**

In vitro propagation of *S. scutellarioides* was carried out by culturing shoot tip explants onto LS, MS and B5 basal medium supplemented with different concentrations of BAP. Comparative analysis of the different types of basal media showed that the LS basal medium supplemented with 8.88 µM BAP was suitable for multiple shoots formation on *S. scutellarioides* (~ 13 shoots/explant). Addition of higher concentrations of BAP (35.5 µM) inhibited multiple shoots and induced abnormal plantlet formation in this species.

Culturing shoot tip explants on B5 medium supplemented with 4.52 µM BAP and 2.26 µM 2,4-D produced pink friable callus. On the other hand, culturing leaf explants on MS and B5 media both supplemented with 4.52 µM 2,4-D and 0.46 µM 2,4-D, produced yellowish and grayish friable calli, respectively. Following calli induction, cell suspension culture of *S. scutellarioides* was successfully initiated on MS medium supplemented with 2.26 µM 2,4-D and 0.47 µM kinetin. The cell suspension required only half the amount of 2,4-D

required for calli induction on solid medium (MS + 4.52 µM 2,4-D + 0.46 µM kinetin).

In an effort towards achieving genetically stable plant tissue culture material, for the future induction of valuable secondary metabolite, adventitious roots and hairy roots were induced from *S. scutellarioides* explants. Quantitative and qualitative assessments of the biomass producing from each of the culture method were analyzed.

Adventitious root cultures in *S. scutellarioides* leaves were induced by placing explants onto MS basal medium supplemented with different concentrations of auxins (NAA, IBA, IAA) and cytokinin (kinetin). Comparative analysis of the roots enhanced showed that MS medium supplemented with 5.0 µM IBA produced rapidly growing adventitious roots. The presence of cytokinin (kinetin) was inhibiting to adventitious root formation in *S. scutellarioides*. Supplementing MS medium with auxin either IBA or NAA was enough to induce adventitious root formation.

Hairy roots of *S. scutellarioides* were induced by inoculation of leaf explants with *A. rhizogenes* strains TR 105, LBA 9402, 8196 and ATCC 15834. These strains showed different abilities to induce hairy root formation in the leaf explants. Assessment of the plant susceptibility to the different *A. rhizogenes* strains showed that the strains ATCC 15834, TR 105, LBA 9402, and 8196 produced 56.3%, 25.5%, 21.5%, and 13.8% transformation efficiencies, respectively. Acetosyringone was found to be useful for enhancement of hairy root production in *S. scutellarioides*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN TISU KULTUR DAN SISTEM PRODUKTIVITI AKAR RERAMBUT TUMBUHAN
***Solenostemon scutellarioides* (L.) Codd**

Oleh

LE VINH THUC

September 2004

Pengerusi: **Profesor Madya Norihan Mohd Saleh, Ph.D.**

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Pembiakan secara *in vitro* dilakukan keatas eksplan pucuk *S. scutellarioides* menggunakan media LS, MS dan B5 yang mengandungi kepekatan BAP berbeza. Perbandingan ke atas media yang digunakan menunjukkan pertumbuhan pucuk berganda *S. scutellarioides* (~ 13 pucuk/eksplan) adalah paling sesuai dalam basal medium LS yang mengandungi 8.88 µM BAP. Penambahan kepekatan BAP yang tinggi (35.5 µM) memberi kesan rencatan terhadap perkembangan pucuk berganda bagi tumbuhan ini.

Pengkulturan eksplan pucuk diatas medium B5 yang mengandungi 4.52 µM BAP dan 2.26 µM 2,4-D menghasilkan kalus berwarna merah muda yang mudah terlerai. Manakala eksplan daun menghasilkan kalus kuning dan mudah terurai diatas MS yang mengandungi 4.52 µM 2,4-D dan 0.46 µM serta kalus kelabu yang mudah terlerai di atas medium B5 dengan kandungan hormon yang sama. Seterusnya ampaian sel *S. scutellerioides* telah berjaya diinisiasikan dalam medium MS yang mengandungi

2.26 µM 2,4-D dan 0.46 µM kinetin. Ampaian sel ini memerlukan hanya separuh sahaja kandungan 2,4-D berbanding dengan medium pepejal bagi mengaruh kalus (MS + µM 2,4-D + 0.46 µM kinetin).

Dalam usaha ke arah memperoehi kestabilan genetik kultur tumbuhan bagi tujuan menghasilkan metabolit sekunder yang bernilai dimasa depan, akar adventitious dan akar rerambut juga telah diaruh daripada eksplan *S. scutellarioides*.

Penghasilan biomass daripada kaedah kultur akar adventitious dan rerambut dinilai secara kuantitatif dan kualitatif ke atas biomass yang dihasilkan menggunakan kedua-dua kaedah pengkulturan telah dilakukan. Akar adventitious *S. scutellarioides* telah diaruh daripada eskplan daun mengunakan medium MS yang mengandungi kepekatan auksin (NAA, IBA, IAA) dan sitokinin (kinetin) berbeza. Analisia menunjukkan medium MS yang mengandungi kepekatan 5.0 µM IBA menghasilkan pertumbuhan akar adventitious paling cepat. Terdapat kesan kerencatan akar adventitious *S. scutellarioides* dengan kehadiran sitokinin (kinetin). Perambahan auksin (IBA, NAA) dalam medium MS sahaja sudah memadai untuk mengaruh penghasilan akar adventitious.

Akar rerambut telah diaruh daripada eskplan daun *S. scutellarioides* dengan menggunakan strain TR105, LBA 9402, 8196 dan ATCC 15834 *A. rhizogenes*. Kesemua strain ini menunjukkan keupayaan berbeza dalam pembentukan akar rerambut. Penilaian sensitiviti tumbuhan ini terhadap

strain *A. rhizogenes* tersebut menunjukkan keberkesanan transformasi 56.3% bagi ATCC 15834, 25,5% bagi TR 105, 21.5% bagi LBA 9402 dan 13.8% bagi 8196 adalah berbeza. Kehadiran asetosringone penting untuk meningkatkan keberkesanan aruhan akar rerambut bagi *S. scutellarioides.*

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I certify that an Examination Committee met on 2nd September 2004 to conduct the final examination of Le Vinh Thuc on his Master of Science thesis entitled "Establishment of Tissue Culture and Hairy Root Production Systems for *Solenostemon scutellarioides* (L.) Codd" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

LE VINH THUC

Date: 20 Sept. 2004

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LIST OF ABBREVIATION

%	Percentage
µM	Micromolar
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	N6-isopentyladenine
A. rhizogenes	*Agrobacterium rhizogenes*
ANOVA	Analysis of variance
Auxin	Plant growth regulator resembling IAA in physiological activity
B5	Gamborg medium
BAP	6-Benzylaminopurine
cm	Centimeter
CPA	p-chlorophenoxyacetic acid
Cytokinin	Plant growth regulator stimulating cell division and resembling kinetin in physiological activity. Mainly N_6 substituted aminopurine compounds.
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleotic acid
EDTA	Ethylenediaminetetraacetic acid
Explant	Excised fragment of plant tissue or organ used to initiate a tissue culture
µg	Microgram
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
Kinetin	6-Furfurylaminopurine
l	Liter
LS	Linsmaier and Skoog
ml	milliliter
mm	Millimeter

MS	Murashige and Skoog
MW	Molecular weight
NAA	∝-Napthtalene acetic acid
°C	Degree centigrade
OD	Optical density
Ri	Root inducing
rpm	Revolution per minute
S. scutellarioides	*Solanostemon scutellarioides*
T-DNA	Transferable-DNA
T_L	Left border of T-DNA
T_R	Right border of T-DNA
Vir	Virulence

CHAPTER 1

INTRODUCTION

Plant secondary metabolites are known to play a major role in the adaptation of plants to their environment. They are also important sources of active pharmaceuticals, food additives, cosmetic industries and pesticides such as rosmarinic acid, vinblastine and ginsenoside. However, useful secondary metabolites distributed in plants are rather more restricted than primary metabolites such as nucleotides, nucleic and amino acids, protein and carbohydrates. In addition, secondary metabolites are found in a few species or even within a few varieties within a species (Alfermann and Petersen, 1995). They are also limited in occurrence in the ornamental plants (Ramawat, 1999a). Rosmarinic acid for example occurs especially within the family of Boraginaceae and the subfamily Nepetoideae within the Labiate (Pedersen, 2000). They mostly accumulate in plant in small quantities, sometimes in specialized cells, making their extraction from the plant often difficult (Sasson, 1991).

Several studies suggested that production of plant secondary metabolites can be improved by using tissue and cell culture techniques (Bourgaud *et al.,* 2001; Suresh *et al.,* 2001; Luczkiewicz *et al.,* 2002). Cell suspension cultures of *Coleus blumei* for example are known to produce rosmarinic acid (Razzaque and Ellis, 1977). Sixty fold increases in anthraquinones

production from 0.3% dry weight to 18% dry weight have been reported in the cell suspension cultures of *Morinda citrifolia* (Misawa, 1994). *In vitro* culture systems such as cell suspension, callus, root, shoot and the transformed 'hairy root' cultures (Scragg, 1995) have been extensively studied with the objective of improving the production of secondary plant compounds (Gontier *et al.*, 1994; Bourgaud *et al.*, 2001).

Plant cell and organ cultures may provide steady and controlled environment for long-term supplies of plant secondary metabolites and protein (Bhadra *et al.*, 1993), enhanced production of pharmaceutically valuable alkaloids (Shanks *et al.*, 1997), and large scale production of secondary metabolites (Tikhomiroff and Jolicoeur, 2002). Cell cultures have higher rates of metabolism than intact differentiated plants because the initiation of cell growth in the culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle. This is the most important advantage of plant cell cultures as model systems for the study of the biosynthetic pathways, as secondary metabolite formation can take place within a short cultivation time, about 2 - 4 weeks (Dörmenburg and Knorr, 1995).

Although undifferentiated cell cultures have been studied, a large research interest has also been shown in the hairy root cultures (Bourgaud *et al.*, 2001). Hairy roots, a result of successful transformation of plant with *Agrobacterium rhizogenes*, is a possible source of secondary metabolites (Berzin *et al.*, 2000) because of the genetic and biochemical stability (Young-Am *et al.*, 2000), high level of differentiation as compared to cell

suspensions, amenability to genetic transformation (Shanks *et al.,* 1997) and rapid growth rate (Hamill and Lidgett, 1997; Shanks and Morgan, 1999). Hairy roots have been successfully applied in the production of some alkaloids (Tikhomiroff and Jolicoeur, 2002) such as ß-carboline glucoid, ruine and serotonin (Misawa, 1994). However the only set-back is the lack of technology on how to scale up the hairy root culture to an industrial scale for a more economical process.

Solenostemon scutellarioides or locally name hati-hati has been known as a source of rosmarinic acid (Razzaque and Ellis, 1977) and also contains high levels of anthocyanins (Misawa, 1994). Rosmarinic acid possesses antioxidative properties (Tada *et al.,* 1996), antibacterial and anti-inflammatory effects (Petersen and Simmonds, 2003). It can be also used to prevent bronchial asthma, apasmogenic disorders, hepatotoxicity, ischaemic heart disease, and cancer disease (Al-Sereiti *et al.,* 1999) and can against human immunodeficiency virus types1 (HIV-1) (Mazunder *et al.,* 1997). Many studies on rosmarinic acid production by callus and cell suspension cultures of *Labiatae* family have been reported (Mizukami *et al.,* 1993; Su and Lei, 1993; Kintzios *et al.,* 1999). However, very limited studies have been carried out on medium optimization of callus induction and cell suspension culture. In addition, there has yet to be any report on *in vitro* propagation of multiple shoot, hairy root and normal root of *S. scutellarioides*. Therefore, the objectives of this research are:

1. To propagate *in vitro* cultures of *S. scutellarioides.*